

Unregulated exposure of the ribosomal M-site caused by NAC depletion results in delivery of non-secretory polypeptides to the Sec61 complex

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Abstract Nascent polypeptide associated complex (NAC) interacts with nascent polypeptides emerging from ribosomes. Both signal recognition particle (SRP) and NAC work together to ensure specificity in co-translational targeting by competing for binding to the ribosomal membrane attachment site. While SRP selects signal-containing ribosomes for targeting, NAC prevents targeting of signal peptide-less nascent chains to the endoplasmic reticulum membrane. Here we show that the ribosome binding that occurs in NAC's absence delivers signal-less nascent chains to the Sec61 complex, underscoring the danger of unregulated exposure of the ribosomal M-site. Recently, the idea that NAC prevents ribosome binding has been challenged. By carefully examining the physiologic NAC concentration in a variety of tissues from different species we here demonstrate that the discrepancy resulted from subphysiologic NAC concentrations.

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Key words: Nascent polypeptide associated complex; Ribosomal membrane attachment site; Sec61 complex

1. Introduction

Various cellular functions require the establishment and maintenance of compartmentalization of proteins and their associated functions within the various membrane-bounded organelles. Therefore, a central problem in establishing compartmentalization is to understand how proteins either during or after their synthesis arrive at their proper cellular destinations [1–4]. Since the biogenesis of most secretory, luminal content, and integral membrane proteins begins with their synthesis on ribosomes bound to the endoplasmic reticulum (ER) membrane, it is important to understand how cells insure that only the right ribosomes, i.e. those translating a secretory protein, bind, and conversely how they prevent ribosomes translating cytoplasmic proteins from binding. Indeed the elucidation of the secretory pathway was initially linked to the observation that a subset of ribosomes is bound to the ER membrane [5].

After it was shown that polypeptides made on these ER-bound ribosomes are discharged into the lumen of the ER, efforts were made to disassemble and then reconstitute the process of ribosome binding *in vitro*. This culminated in the development of a widely used system where *in vitro* translation extracts prepared from either wheat germ or rabbit retic-

ulocyte lysates are supplemented with canine pancreas rough microsomes [6].

Using this assay system, the signal recognition particle (SRP) was identified as a factor that is required for co-translational targeting to occur. SRP binds signal peptides as they emerge from the ribosome, and subsequently engages its receptor at the ER membrane [2]. The requirement for SRP for ribosome-membrane interaction was, in our minds, difficult to reconcile with the observation that purified non-translating 80S ribosomes removed from cytosolic proteins bind to translocation sites on ER membranes without an apparent need for SRP [7–9]. Given this affinity of ribosomes for ER membranes, an additional question is how the cell prevents ribosomes synthesizing cytosolic proteins from inappropriately binding to ER membranes. We therefore reasoned that other cytosolic factors may modulate ribosome binding.

We discovered nascent polypeptide associated complex (NAC) as a cytosolic heterodimeric factor that crosslinks to regions of nascent polypeptides as they emerge from the ribosome [10,11]. Because it acts at the interface of the ribosome and the cytosol, we hypothesized that NAC is in a unique position to modulate ribosome binding. To test this idea we modified the standard ribosome binding assay in which membrane-bound and free ribosomes are separated by floatation in sucrose density gradients so that we could examine the binding of ribosome nascent chain complexes (RNCs) to ER membranes and also assess the effects of factors such as NAC and SRP on RNC binding.

Using this assay we showed that ribosome binding alone, without the participation of cytosolic factors, can deliver nascent secretory proteins to translocation sites such that they are efficiently translocated across the ER membrane when released from the ribosome. Surprisingly, we also observed that nascent chains lacking signal peptides were efficiently targeted, but translocated inefficiently [12]. Addition of only SRP did not increase the targeting efficiency of RNCs. Purified NAC alone blocked the binding of RNCs whether or not they harbored signal peptides as well as 80S ribosomes [29]. When both NAC and SRP were present, only signal peptide-containing RNCs were targeted [13]. From these studies we concluded that ribosome binding can efficiently target nascent chains for translocation, but that the specificity of ribosome binding which is normally observed results from the combined action of NAC and SRP. Thus our experiments with RNCs are consistent with all previous data and specifically explain why SRP is required for translocation in the wheat germ translation system, namely because NAC is also present in these lysates.

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We envision that ribosomes bind to translocation sites via a membrane attachment site (M-site), and that NAC prevents ribosome binding by blocking the M-site unless a signal peptide emerges from the ribosome and comes to lie in the vicinity of the M-site. Under these circumstances, as SRP has a very high affinity for signal peptides, it successfully competes with NAC for occupancy of the M-site. Indeed, we have shown that in the absence of SRP receptor, SRP blocks ribosome binding, suggesting that SRP transiently occupies the M-site to keep it free of NAC [29]. When the ribosome nears the membrane, the SRP receptor displaces SRP from the M-site so that the ribosome can engage the translocation site [13]. Consistent with our model that NAC and SRP compete for occupancy of the M-site, others have independently shown that NAC and SRP compete for binding to a common site on RNCs [14,15]. We have further shown that the interaction of the ribosome with the Sec61 complex is inhibited by NAC.

Recently, two reports challenged the idea that NAC prevents ribosome binding [16,17]. The experiments in question were executed using a modified version of the *in vitro* system utilized by us and others where the microsomal membranes were stripped of their endogenous ribosomes with puromycin rather than EDTA. The fact that this modified system also does not require SRP for RNC binding suggests that puromycin/high salt stripped microsomes (PKRMs), under the conditions utilized, do not faithfully reconstitute the *in vivo* situation.

Here we show that in the absence of NAC ribosome associated signal-less nascent chains are in direct contact with the Sec61 complex, the central component of the translocon, an observation that underscores why cells must prevent this inappropriate ribosome binding from occurring. Since we suspected that the aforementioned investigators failed to detect NAC activity because of insufficient NAC concentrations utilized, we determined the physiologic NAC concentrations in a wide variety of tissues and organisms and show that when NAC is present near its physiologic concentration it does block ribosome binding.

2. Materials and methods

2.1. *In vitro* transcription and translation, isolation of RNCs, and RNC targeting assay

In vitro transcription and translation of truncated mRNAs was as described [18]. Ribosome/nascent chain complexes were prepared and targeting assays were performed exactly as described [12]. Photocross-linking, where trifluoromethyldiazirino benzoic acid (TDBA) modified lys-tRNA was added to a reticulocyte lysate translation system [19], was according to Görlich et al. [20]. PKRMs were prepared as described [26].

2.2. Preparation of recombinant NAC

The genes encoding glutathione *S*-transferase fusion proteins with both α and β NAC were expressed in *Escherichia coli*. The fusion proteins were purified separately and the GST portion was removed by cleavage with thrombin. The subunits were reconstituted into heterodimeric NAC. The complex was shown to be functionally active in our standard assays [29] (Beatrix et al., in preparation, and see Fig. 1b).

2.3. Determination of NAC concentration in various tissues

Measured weights of the tissues indicated in Fig. 2 were frozen in liquid nitrogen and ground in four volumes of 20 mM Tris buffer, pH 7.5. Samples were treated with DNase and RNase for 10 min at 37°C prior to the addition of SDS to 3%. After heating to 75°C for 30 min, lysates were cleared by centrifugation. The protein concentrations of

the lysates were determined [27] and ranged from 125 (*Drosophila* and lung) to 500 mg/ml (reticulocytes). These calculations assume that 1 g of tissue occupies a volume of 1 ml. The signals obtained by Western blotting with affinity purified anti- α NAC antibodies were compared to those obtained using recombinant NAC as a standard. Similar results were obtained using anti- β NAC antibodies (not shown).

2.4. Preparation of a concentrated wheat germ cytosol

A wheat germ translation extract [21] was adjusted to 500 mM KOAc and the stripped 80S ribosomes and subunits were sedimented by centrifugation (TLA 100.1 rotor, 100 000 rpm, 30 min, 4°C). The post-ribosomal supernatant was adjusted to 60% ammonium sulfate and centrifuged. The resulting pellet was resuspended in RBB and dialyzed. NAC and total protein were concentrated about 2.5-fold by this procedure.

3. Results

First we assayed the ability of NAC to block the binding of RNCs to microsomes that had been stripped of their endogenous ribosomes either by EDTA/KOAc (EKRM) or puromycin/KOAc (PKRM) treatment (Fig. 1a). RNCs were generated by *in vitro* translation in rabbit reticulocyte system of a mRNA lacking a stop codon and encoding the first 77 residues of the signal peptide-less peroxisomal firefly luciferase (77aaffLuc). Because termination of translation cannot occur, nascent chains remain stably associated with the ribosomes. Associated cytosolic factors such as NAC and elongation factors are removed from the RNCs by high salt stripping, and RNCs are collected by sedimentation. These high salt stripped 77aaffLuc RNCs were incubated with the indicated concentrations of purified NAC prior to the addition of either EKRM or PKRM. Membrane-bound and free RNCs were separated by centrifugation in discontinuous sucrose density gradients. Membranes containing bound RNCs float up in these gradients and are recovered in the top fractions of the gradients, whereas unbound RNCs remain in the bottom fractions. NAC effectively blocks the binding of the 77aaffLuc RNCs to the EKRM, and although inhibitory, is less effective in blocking binding to PKRMs (Fig. 1a).

Because Neuhof et al. [16] and Raden and Gilmore [17] found SRP-independent targeting, a phenomenon that we observed occurring when NAC concentrations are low, we surmised that the NAC concentrations were subphysiologic in these experiments. We therefore investigated whether increasing the NAC concentration could block binding to PKRMs. Using NAC purified from bovine brain we were not able to add NAC to a final concentration greater than 1.25 μ M, and were therefore not able to determine whether higher concentrations would further reduce RNC binding to PKRMs. To circumvent this limitation we made highly concentrated recombinant NAC (rNAC) which is active in the crosslinking assay that was utilized in the identification and purification of NAC (see Fig. 4a) and prevents the association of RNCs with EKRM (Beatrix et al., in preparation). As is shown in Fig. 1b, recombinant NAC is as effective as bovine brain NAC in preventing the binding of 77aaffLuc RNCs to PKRMs, and increased concentrations of NAC effectively block RNC binding to near background levels.

We next determined the concentration of NAC in various murine tissues, rabbit testis and reticulocyte lysates, in *Drosophila*, and in wheat germ to determine whether the concentrations of NAC utilized in Fig. 1b were within the physiologic range. 40 μ g of protein from each tissue (100 μ g in the

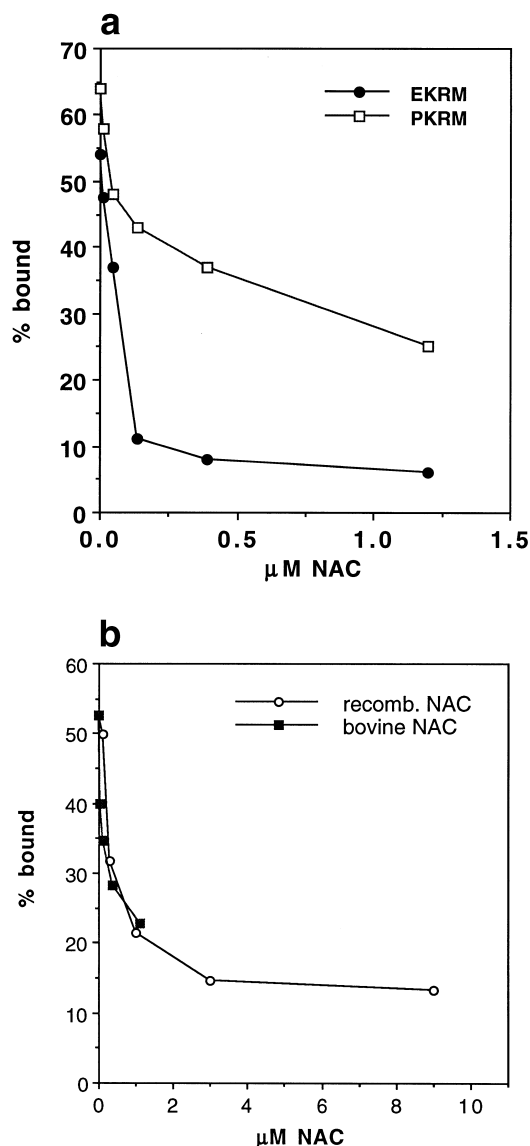


Fig. 1. NAC blocks RNC binding to PKRMs and EKRM. a: High salt stripped 77aaffLuc RNCs were prepared in a reticulocyte lysate translation system supplemented with [^{35}S]Met. 1.5 μl RNCs (102000 cpm/ μl) were incubated with the indicated concentrations of purified NAC for 2 min at 26°C and 5 min on ice. After the addition of 2 eq. PKRMs or EKRM, samples were subjected to a second round of incubation. Final assay volumes were 20 μl . Binding was assessed using the flotation assay. b: High salt stripped 77aaffLuc RNCs were incubated with purified bovine or recombinant NAC at the indicated concentrations. Samples were incubated for 2 min at 26°C and 5 min on ice. 1 eq. PKRMs was added, and samples were incubated as above before being assayed for RNC binding with the flotation assay.

case of the reticulocyte lysate) was analyzed by Coomassie staining (Fig. 2a) or by Western blotting (Fig. 2c). We calculated the concentration of NAC in murine lung and spleen (Fig. 2b) and estimate it to be 3 μM and 5 μM . The NAC concentrations in the other tissues tested are similar (Fig. 2c). Importantly, the concentrations at which NAC effectively blocks binding to both EKRM and PKRM are well within the physiologic range.

The experiments in Fig. 1 were performed using mammalian RNCs and mammalian NAC. Since some of the experi-

ments questioning NAC's role in blocking ribosome binding were performed using wheat germ RNCs with mammalian PKRMs, we wished to determine whether wheat germ NAC functions to prevent wheat germ RNCs from binding to PKRMs. Wheat germ 77aaffLuc RNCs were synthesized, but in contrast to the foregoing experiments, the RNCs were not stripped or fractionated away from the cytosol. Instead, 1.5 eq. PKRMs was added into a 16 μl translation before analysis with the RNC binding assay (Fig. 3, lanes 1 and 2). About half of the RNCs bound despite the presence of NAC. This result is similar to what was observed by those who dispute NAC's role in inhibiting RNC binding. Since this inappropriate binding does not normally occur in living cells, we reasoned that the inability of NAC, or perhaps any other factor, to block inappropriate ribosome binding resulted from a peculiar and non-physiologic aspect of the *in vitro* system. Inappropriate binding of signal-less RNCs was not observed if EKRM are utilized (not shown), indicating a difference between EKRM and PKRM.

Since the final protein concentration in our wheat germ translation mixtures is approximately 15 mg/ml and we extracted 390 mg from 1 g (1 ml) of floated wheat germ, we estimated that the NAC concentration in our assay is not higher than 0.5 μM . We investigated whether restoration of bulk cytosolic protein including NAC close to physiologic concentrations would prevent the inappropriate binding of 77aaffLuc RNCs. To this end, we first depleted wheat germ

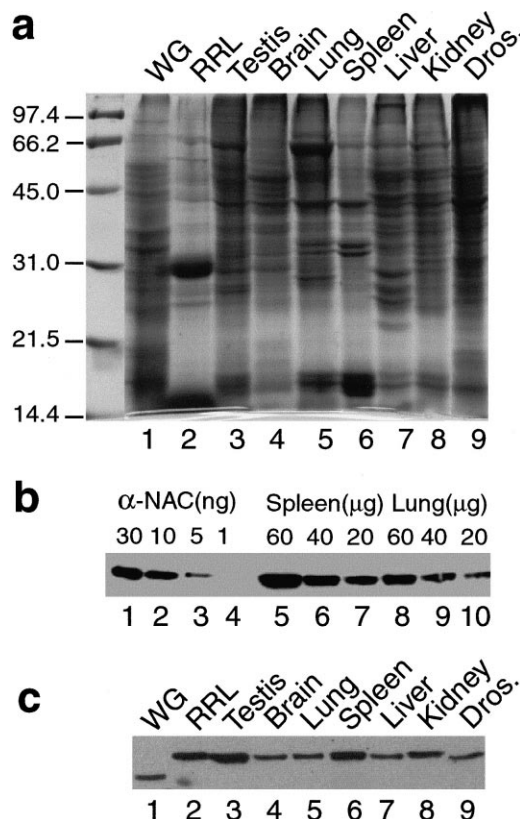


Fig. 2. NAC is an abundant cellular complex. a: Coomassie staining of 40 μg (except for reticulocyte lysates where 100 μg was loaded) tissue lysates. b: The indicated amounts of recombinant αNAC or spleen and lung lysates were analyzed by Western blotting as described in the text. c: Comparison of NAC levels in various tissues as assessed by Western blotting. The wheat germ NAC migrates more rapidly than the mammalian protein [10].

lysates of their ribosomes by sedimentation under high salt conditions. The post-ribosomal supernatant was subjected to ammonium sulfate precipitation and dialysis. The resulting fraction containing wheat germ NAC represented a source of concentrated cytosol. As shown in Fig. 3, reversing the dilutional effect by addition of the concentrated cytosol prevents inappropriate binding. Using semiquantitative Western blotting we estimate that the final, maximal NAC concentration (lanes 7 and 8) is 2–3 μ M (not shown), near the physiologic NAC concentration (see Fig. 2b,c). The variability in translation efficiency observed (e.g. compare Fig. 3, lanes 1 and 2 with 5 and 6) resulted from the effect of adding the bulk protein to the assays. Therefore, one must examine the samples for the proportion of RNCs bound rather than the absolute amount of binding. We also confirmed that the addition of bulk protein does not release the nascent chains from the ribosomes (not shown).

To verify that the added recombinant NAC was actually associating with the RNCs thereby preventing ribosome binding and subsequent insertion of the nascent chains into the translocons, we utilized a crosslinking technique that allow for determining which factors are intimately associated with the nascent chain at a particular instant [20].

In this approach the ϵ -amino group of lys-tRNA is modified with TDBA, a photoactivatable, irreversible crosslinking reagent. When included in in vitro translation/translocation systems, the lysine residues bearing photoactivatable crosslinkers are incorporated into nascent polypeptides as dictated by the positions of the lysine codons in the mRNA. Photoadducts formed between the nascent chain and proteins are evident by observing decreased electrophoretic mobility of the radiolabeled nascent chain.

High salt stripped reticulocyte lysate 77aaffLuc RNCs were prepared with TDBA-lys-tRNA. After incubation with the indicated amounts of rNAC, PKRMs were added and samples were subjected to centrifugation in the usual manner. Samples were irradiated to induce crosslinking prior to collecting the top ('T') and bottom ('B') fractions. The pattern of crosslinks in each fraction was analyzed by SDS-PAGE and fluorography (note that the nascent chain content could not be estimated by fluorography because the film is overexposed for the purposes of seeing the 77aaffLuc in order to allow for visualization of the fainter crosslinked bands).

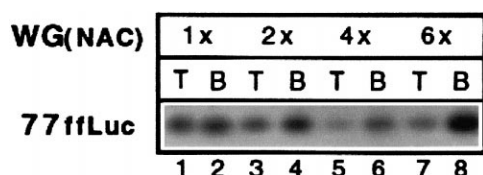


Fig. 3. Subphysiologic cytosol concentrations result in inappropriate targeting of signal peptide-less RNCs to PKRMs. A wheat germ translation assay programmed with 77aaffLuc mRNA was supplemented with a concentrated post-ribosomal wheat germ cytosol prepared as discussed in Section 2. The reaction in lanes 1 and 2 was not supplemented and lanes 3–8 were supplemented with the indicated cytosol concentrations. After translation for 20 min at 26°C, 1.5 eq. PKRMs were added to a 6 μ l translation assay and targeting was allowed to occur for 2 min at 26°C. Samples were directly analyzed for RNC binding using the floatation assay. The concentration designations (2 \times , 4 \times , 6 \times) refer to the final concentrations of bulk cytosol compared to the standard working concentrations of our wheat germ lysates. The final, maximal NAC concentration used is 2–3 μ M.

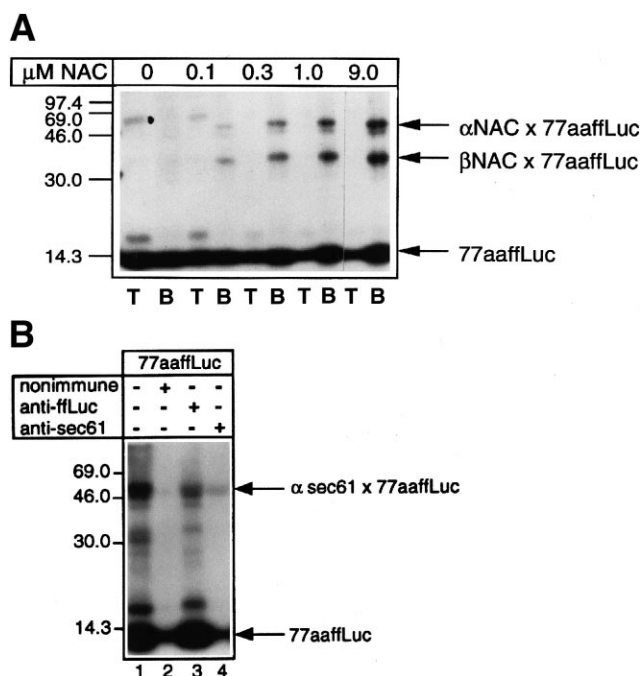


Fig. 4. Probing the molecular environment of the nascent chain revealed with photocrosslinking. A: High salt stripped 77aaffLuc RNCs prepared with TDBA-lys-tRNA and [35 S]Met were incubated with the indicated concentrations of recombinant NAC prior to the addition of 1 eq. PKRMs. After centrifugation, samples were irradiated to induce crosslinking, and were then divided into top (T) fractions containing bound RNCs and bottom (B) fractions containing free RNCs. In the absence of NAC, 77aaffLuc nascent chains crosslinked to Sec61 α p (marked with a dot, and see B). Addition of NAC results in loss of the Sec61 α p crosslink and appearance of α and β NAC crosslinks. Note that the crosslinks to membrane proteins are found in top fractions, whereas crosslinks to NAC are only found in bottom fractions with the unbound RNCs. Assay conditions were identical to those in Fig. 1b. B: A sample identical to the one shown in the first lane of A was prepared and divided into four equal aliquots (lane 1) and subjected to immunoprecipitation with non-immune serum (lane 2), an antiserum against ffLuc (lane 3), or an antiserum against a Sec61 α p peptide (lane 4) to confirm that the observed photoadduct represents a crosslink between the 77aaffLuc and Sec61 α p.

When no NAC is present, a fraction of the targeted nascent chains in the top fraction crosslinked to a protein with an approximate molecular weight of 35–40 kDa (giving rise to a ca. 55 kDa photoadduct, marked with a dot in Fig. 4A) which we identified by immunoprecipitation (Fig. 4B) as Sec61 α p, the central integral membrane protein of the translocon. As expected, the crosslink is only apparent in the top fractions where the membranes are found. When 0.1 μ M NAC was added before the addition of the PKRMs and crosslinking, the intensity of the Sec61 α p crosslink diminished, indicating that fewer RNCs arrived at translocation sites. Concomitantly, crosslinks to both the α and β subunits of NAC appeared. As expected, these crosslinks were found exclusively in the bottom fractions with the unbound/free RNCs. Thus, by binding to the RNCs, NAC prevents their membrane association. As more NAC was added, more bound to the RNCs, and fewer RNCs bound to the membranes (see also Fig. 1a).

Taken together these results show that by binding to RNCs, NAC prevents their association with PKRMs. The ability of the signal-less 77aaffLuc nascent chain to interact with

Sec61 α p shows that ribosome binding can direct nascent chains to the core of the translocon in a non-specific manner, and underscores the need to prevent inappropriate ribosome binding from occurring.

In addition to the Sec61 α p crosslink, a photoadduct of approximately 25 kDa, which represents a crosslink between the 77aaffLuc nascent chain and an unidentified ca. 10 kDa protein is evident. As the crosslink resists extraction with sodium carbonate and is not released by treatment with phospholipase A₂ (not shown), the crosslink represents an integral membrane protein.

4. Discussion

We have shown that the discrepancy between our previously published data and those of others regarding NAC's effect on RNC binding most likely resulted from the use of PKRMs rather than EKRMs and from an insufficient, sub-physiologic NAC concentration. Nevertheless, when NAC is present at its normal, physiologic concentration, it prevents RNC binding to PKRMs. Further evidence for a central role of NAC in cell physiology is that *Drosophila* and mice lacking NAC gene expression have an early embryonic lethal phenotype [22,23].

A potential pitfall of biochemical systems which are necessarily diluted when compared to in vivo cytosolic concentration is studying the effects of abundant proteins with low affinities, as is the case with NAC. Another demonstration of this phenomenon is the involvement of Hsp70 in protein targeting. Initially, biochemical assays failed to detect the participation of this factor in protein targeting. Only after genetic studies revealed a role for Hsp70 were the biochemical systems modified to reveal the function of Hsp70 [24,25].

In the PKRM based system it is not only difficult to detect NAC activity, but SRP independent targeting occurs [16,17]. It has long been known, however, that RNC targeting in wheat germ systems supplemented with KRM is SRP dependent [28]. Since we had previously observed that SRP independent targeting occurs at insufficient NAC concentrations [13], we performed the experiments above to show that replenishing NAC inhibits RNC binding. As a control we also determined that targeting of signal containing RNCs again becomes SRP dependent upon readdition of NAC (not shown), consistent with published data [13,14].

These investigators who describe SRP independent targeting also claim that while SRP is not required for targeting, it may help to increase targeting efficiency. We suggest that the observed enhancement by SRP was probably due to overcoming the block on RNC binding resulting from residual NAC.

Both of the reports that failed to detect NAC activity do describe ribosome mediated targeting, just as we have previously done [12]. A central problem is that they observed inappropriate ribosome binding which does obviously not normally occur. In contrast to the established system using EKRM, their system does not reflect what occurs in vivo. Indeed, SRP could not have been discovered in this assay system. The crosslink of the signal peptide-less 77aaffLuc to Sec61 α p, part of the central component of the translocon [26] which is observed upon NAC depletion underscores the importance of preventing such binding. Consistent with this finding we have recently shown that NAC directly blocks

the interaction of the ribosomal M-site with the Sec61 complex [29]. If no alternative factor is found that prevents inappropriate ribosome binding in the PKRM based in vitro system, it must be considered deficient. We think that the data they presented are valid, but misinterpreted. The observation of inappropriate RNC binding and SRP independent binding should suggest that an activity which is normally present is missing. Here we have again demonstrated that the missing factor is NAC, and that both NAC and SRP are required for guaranteeing the normally observed specificity in ribosome binding and the subsequent specificity of entry into the secretory pathway.

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